PB81-222234

Mutagenesis and Morphological Transformation of Mammalian Cells by a Non-Bay-Region Polycyclic Cyclopenta(cd)pyrene and Its 3,4,-Oxide 1

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

27 Aug 80

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
Please read Instructions on the reverse before completing)

MS-80-020

1. REPORT NO. EPA-600/J-80-304	2. JOURNAL ARTICLE
3. RE PB	PB81-222234
4. TITLE AND SUBTITLE: Mutagenesis and Morphological Transformation of Mammalian Cells by a Non-Bay-Region Polycyclic Cyclopenta(cd)pyrene and Its 3,4-Oxide	5. REPORT DATE: July 1981
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): A. Gold, S. Nesnow, M. Moore, H. Garland, G. Curtis, B. Howard, D. Graham, and E. Eisenstadt	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Genetic Toxicology Division, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	10. PROGRAM ELEMENT NO. ACSL1A 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Office of Research and Development (RTP,NC), Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVE 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Cancer Research 40: 4482-4484, 1980

16. ABSTRACT

Cyclopenta(cd)pyrene, a constituent of environmental emissions, has been found to mutate and transform mammalian cells in culture. Cyclopenta(cd)pyrene 3,4-oxide, a presumed metabolite, was found to be a direct-acting mutagen and to transform mammalian cells. These results suggest that cyclopenta(cd)pyrene 3,4-oxide may be an ultimate mutagenic form of the parent hydrocarbon.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Grou

PRODUCT OF:
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT: RELEASE TO PUBLIC	19. SECURITY CLASS (This Report): UNCLASSIFIED	21. NO. OF PAGES
	20. SECURITY CLASS (This page): UNCLASSIFIED	22. PRICE

[CANCER RESEARCH 40, 4482–4484, December 1980]
0008-5472/80/0040-0000$02.00

EPA-600/J-80-304
Journal Article

Mutagenesis and Morphological Transformation of Mammalian Cells by a Non-Bay-Region Polycyclic Cyclopenta(*cd*)pyrene and Its 3,4-Oxide[1]

Avram Gold, Stephen Nesnow,[2] Martha Moore, Helen Garland, Gaynelle Curtis, Barry Howard, Deloris Graham, and Eric Eisenstadt

Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514 [A. G.]; Genetic Toxicology Division (MD 68), Health Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, North Carolina 27711 [S. N., M. M., H. G., G. C., B. H., D. G.]; and Department of Microbiology, Harvard School of Public Health, Boston, Massachusetts 02115 [E. E.]

ABSTRACT

Cyclopenta(*cd*)pyrene, a constituent of environmental emissions, has been found to mutate and transform mammalian cells in culture. Cyclopenta(*cd*)pyrene 3,4-oxide, a presumed metabolite, was found to be a direct-acting mutagen and to transform mammalian cells. These results suggest that cyclopenta(*cd*)pyrene 3,4-oxide may be an ultimate mutagenic form of the parent hydrocarbon.

INTRODUCTION

The metabolism and mutagenicity of PAH[3] have been the focus of considerable interest because of their widespread environmental distribution and their potential for carcinogenic activity in humans (5). The biological activity of PAH is dependent on the metabolism of the parent compounds by mixed-function oxidase systems into reactive oxides capable of forming covalent adducts with DNA. As a consequence, considerable effort has been devoted to characterization of the metabolism of these agents and the assessment of the relative biological activities of the metabolic intermediates.

Many biologically active polycyclics have an important geometric feature in common. They contain a bay region which is involved in mutagenic activity.

CPP, (Chart 1), a PAH without a bay region, is a widespread environmental contaminant found in soot (6, 12, 19, 20), cigarette smoke (18), and automobile exhaust (10) and is biologically active. CPP is highly mutagenic with activation in the Ames assay (4) and tumorigenic in mice (13, 21). Despite its potent mutagenicity compared to B(a)P in the bacterial assay, CPP was recently shown to be considerably less active than B(a)P as a mouse skin tumor initiator (21).

A putative ultimate mutagenic-carcinogenic metabolite, CPP 3,4-oxide (Chart 1), has been synthesized and shown to be an extremely potent direct-acting mutagen in the Ames assay (7).

This paper reports results of *in vitro* bioassays on CPP and CPP 3,4-oxide in 2 mammalian systems: mutagenicity in L5178Y mouse lymphoma cells; and morphological transformation in C3H10T½ CL8 mouse embryo fibroblasts.

MATERIALS AND METHODS

***In Vitro* Mammalian Mutagenesis Assay.** The $TK^{+/-}$ L5178Y mouse lymphoma mutagen assay developed by Clive *et al.* (2, 3) to identify mutagens which induce genetic damage at the TK locus was performed according to published methods using Fischer's medium. Trifluorothymidine (1 μg/ml) was used to select for TK-deficient mutants (3). CPP (8) (dissolved in DMSO) was tested in 3% horse serum at a cell concentration of 0.6×10^6 cells/ml with and without an Aroclor 1254-induced rat hepatic S-9 activation system (2). The CPP 3,4-oxide (7) (dissolved in DMSO) was tested in 10% horse serum at a cell concentration of 0.6×10^6 cells/ml without activation. The positive controls, ethyl methanesulfonate and 2-acetylaminofluorene, were dissolved in 0.85% NaCl solution and DMSO, respectively. Two days were allowed for the expression of newly induced mutants.

Morphological Transformation Assay. The mouse embryo fibroblast cell line C3H10T½ CL8, derived by Reznikoff *et al.* (15, 16) and kindly donated by Dr. Charles Heidelberger, was used in these experiments. Cell cultures were incubated in humidified incubators with an atmosphere of 5% CO_2 in air at 37°. All cultures were grown in Eagle's basal medium with Earle's salts and L-glutamine supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The cells were routinely checked for *Mycoplasma* contamination and found to be *Mycoplasma* free.

For transformation, cells were seeded onto 6-mm Petri dishes (1000/dish) in 5 ml in medium (12 dishes/experimental point) and were treated 24 hr later with the hydrocarbon dissolved in acetone (25 μl). After an additional 24 hr, the medium was removed, and the cells received fresh complete medium containing penicillin (100 units/ml) and streptomycin (50 μg/ml). Medium was changed weekly until the cells reached confluency, whereupon the fetal calf serum concentration was reduced to 5%.

At the end of 6 weeks, the dishes were washed with 0.85% NaCl solution, fixed with methanol, stained with Giemsa, and scored for morphological transformation. Three different types of foci have been described after treatment of C3H10T½ CL8 cells with polycyclic hydrocarbons. Only type II and type III foci are scored in this assay, since it has been demonstrated that these foci produce fibrosarcomas upon injection into irradiated C3H mice 33 and 67% of the time, respectively (15, 16).

Control cultures received the appropriate solvent and were treated in the same manner as the exposed cells. Cytotoxicity assays were performed concurrently with the transformation assays and performed using the same protocol, except that the dishes were plated with 200 cells (6 dishes/experimental point) and stained 10 to 12 days later.

JUN 16 1981

RESULTS AND DISCUSSION

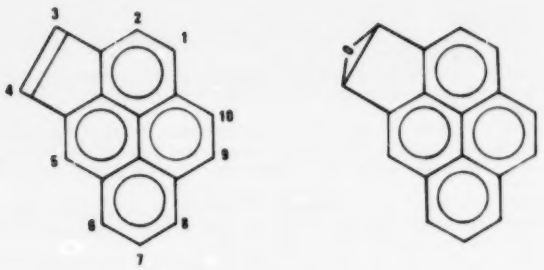
The $TK^{+/-}$ L5178Y mouse lymphoma mutagen assay has

[1] These studies were supported in part by Grants 1-R01-ES02021-01 and 1-R0-1-CA 28622-01 from the USPHS and Grant IN-15U from the American Cancer Society.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; CPP, cyclopenta(*cd*)pyrene; B(a)P, benzo(*a*)pyrene; CPP 3,4-oxide; cyclopenta(*cd*)pyrene 3,4-oxide; TK, thymidine kinase; CL8, clone 8; DMSO, dimethyl sulfoxide.

Received March 4, 1980; accepted August 27, 1980.



I II

Chart 1. Structures for CPP (*I*) and CPP 3,4-oxide (*II*).

been used to quantitate the induction of mutation by a number of diverse chemical agents (2). These cells lack the ability to activate promutagens. Therefore, CPP was tested both with and without metabolic activation to confirm that its mutagenicity would exhibit the dependence on activation typical of other PAH. As expected, CPP was not mutagenic without activation over a concentration range of 0.75 to 30 μg/ml (data not shown).

However, with Aroclor 1254-induced hepatic S-9 activation, CPP was mutagenic (Table 1), giving approximately a doubling of the mutant frequency over control, a result similar to that found in human diploid lymphoblasts (17). The 3,4-oxide was tested in the absence of metabolic activation to determine whether it could be an ultimate mutagen, and it was found to be highly mutagenic in L5178Y cells over a dose range similar to that of CPP with activation. The 3,4-oxide is 2 to 6 times more potent than the parent hydrocarbon over the 70 to 20% survival range. On a molar basis, CPP 3,4-oxide is approximately 600 times as potent as the positive control, ethyl methanesulfonate. These data are consistent with the hypothesis that CPP 3,4-oxide may be an ultimate mutagenic metabolite of CPP.

The C3H10T½ CL8 morphological transformation bioassay responds to a variety of chemical carcinogens including PAH (15). C3H10T½ CL8 mouse embryo cells contain cytochrome P-450 mixed-function oxidase, epoxide hydrase, and water-soluble conjugating enzymes necessary to metabolize, activate, or detoxify chemical carcinogens, especially PAH (14). As shown in Table 2, CPP produced a dose-related response in the formation of both type II and type III transformed foci. At the highest dose of CPP tested, every plate contained at least 1 type III focus. In accordance with the recent report that CPP is a weaker tumor initiator than B(a)P (21), CPP was found to be less active than B(a)P in the C3H10T½ CL8 transformation assay (Table 2).

CPP 3,4-oxide transforms C3H10T½ CL8 cells at concentrations of 0.3 μg/ml. At 3 μg/ml, it produces both type II and III foci with no major concomitant toxicity. The lack of toxicity is remarkable in that structurally related K-region oxides of B(a)P and 3-methylcholanthrene are highly cytotoxic (data not shown). The diol-epoxide metabolite of B(a)P *anti*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene 9,10-oxide transforms these cells and forms covalent adducts with the cellular DNA of these mouse embryo fibroblasts (1).

The potent direct-acting mutagenicity of the 3,4-oxide in both bacterial and mammalian assays, its ability to transform C3H10T½ CL8 cells, and the identification of 3,4-dihydro-3,4-dihydroxycyclopenta(*cd*)pyrene as a major metabolite (9) in Aroclor 1254-induced S-9 metabolism of CPP is strong evidence that CPP 3,4-oxide is both a primary product of enzymatic oxidation and an ultimate mutagen. Further support for this conclusion is the report that the addition of epoxide hydrase to a purified reconstituted monooxygenase activation system drastically reduces the mutagenicity of CPP (21). The effect of epoxide hydrase can be readily explained if CPP 3,4-oxide is the ultimate mutagen and, like other arene oxides (5, 11), is a good substrate for epoxide hydrase. The lower activity of CPP 3,4-oxide in C3H10T½ CL8 cells, when compared to L5178Y cells, is probably a reflection of the ability of the C3H10T½ CL8 mouse embryo cell to further metabolize CPP 3,4-oxide to inactive forms. This type of metabolic deactivation of PAH metabolites has already been demonstrated in C3H10½ CL8 cells (14). Mouse lymphoma cells do not have this metabolic capability (2).

An important distinction between B(a)P diol-epoxide and CPP 3,4-oxide is that the latter is an arene oxide. The epoxidized bond is adjacent to the aromatic nucleus at both termini. The arene oxides, 3-methylcholanthrene 11,12-oxide and B(a)P 4,5-oxide, both fail to transform C3H10T½ CL8 cells (data not shown). CPP is the first arene oxide reported to

Table 1

Mutagenesis of TK$^{+/-}$ L5178Y mouse lymphoma cells by CPP and CPP 3,4-Oxide

TK$^{+/-}$ 3.7.2C L5178Y mouse lymphoma cells were treated with CPP, CPP 3,4-oxide, or 2-acetylaminofluorene dissolved in DMSO or ethyl methanesulfonate dissolved in 0.85% NaCl solution for 4 hr. Following treatment, the media was removed and replaced with fresh media. After a 2-day expression period, cells were cloned for trifluorothymidine resistance (TK-deficient mutants) and viability (see Refs. 2 and 3 for complete protocol).

Concentration (μg/ml)	Total viable clones	Total mutant clones	Total survival (% of control)[a]	Mutant frequency (× 10^6)
CPP (with S-9 activation)				
1.20	439	263	110	120
1.30	365	317	74	172
1.40	436	300	56	138
1.50	407	284	44	139
1.60	399	375	20	188
AAF[b] (30)	270	785	31	581
DMSO (1%)	453	178	100	79
CPP 3,4-oxide				
0.60	536	783	71	292
0.70	472	803	63	340
0.84	536	912	72	340
0.96	463	776	58	335
1.08	475	1234	52	519
1.20	476	1065	44	448
1.32	406	1174	32	578
1.44	341	1090	22	640
1.56	360	1423	23	790
1.68	459	1118	36	487
1.80	273	1204	10	882
2.04	333[c]	1214	6	1460
DMSO (1%)	623	297	100	95
EMS (500)	259	1638	25	1267
Untreated control	558	220	100	79

[a] Survival calculations described by Clive and Spector (3) combine both relative growth in suspension and relative plating efficiency.

[b] AAF, 2-acetylaminofluorene; EMS, ethyl methanesulfonate.

[c] Cells cloned at a density of 12 cells/ml; all other cultures cloned at 6 cells/ml to determine viability.

Table 2

Morphological transformation of C3H10T½ CL8 cells by CPP and CPP 3,4-oxide

C3H10T½ CL8 mouse embryo fibroblasts were treated with CPP or CPP 3,4-oxide (dissolved in acetone) for 24 hr. The media were removed and replaced with fresh media containing antibiotics. After 10 days, the plates were scored for toxicity and, after 6 weeks, for morphological transformation. See "Materials and Methods" for details.

Concentration (μg/ml)	Plating efficiency (%)	No. of type II foci/total dishes	No. of type III foci/total dishes	Dishes with type II and III foci (%)
CPP				
0.01	27	0/12	2/12	17
0.03	28	1/12	1/12	17
0.1	26	0/12	1/12	8
0.3	27	3/12	2/12	25
1.0	29	4/11	7/11	73
3.0	29	6/12	7/12	75
10.0	22	13/12	12/12	100
CPP 3,4-oxide				
0.001	32	2/12	0/12	8
0.003	31	1/11	0/11	9
0.01	30	0/12	0/12	0
0.03	32	1/11	0/11	9
0.1	32	1/12	0/12	8
0.3	32	0/12	2/12	17
1.0	31	0/12	2/12	17
3.0	27	1/12	2/12	25
Acetone (0.5%)[a]	30	2/24	0/24	8
B(a)P[a] (11.0)	18	38/24	59/24	100

[a] Results from 2 separate experiments combined.

transform these cells.

Other metabolites of CPP have been observed from incubations with rat liver homogenates indicating additional pathways of metabolism (9). Studies on the metabolism of CPP in C3H10T½ CL8 cells are in progress to identify other potentially mutagenic and transforming CPP metabolites.

REFERENCES

1. Brown, H. S., Jeffrey, A. M., and Weinstein, I. B. Formation of DNA adducts in 10T½ mouse embryo fibroblasts incubated with benzo(*a*)pyrene or dihydrodiol oxide derivatives. Cancer Res., *39*: 1673–1677, 1979.
2. Clive, D., Johnson, K. O., Spector, J. F. S., Batson, A. G., and Brown, M. M. M. Validation and characterization of the L5178Y/TK$^{+/-}$ mouse lymphoma mutagen assay system. Mutat. Res., *59*: 61–108, 1979.
3. Clive, D., and Spector, J. F. S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutat. Res., *31*: 17–29, 1975.
4. Eisenstadt, E., and Gold, A. Cyclopenta(*cd*)pyrene: a highly mutagenic polycyclic atomatic hydrocarbon. Proc. Natl. Acad. Sci. U. S. A., *75*: 1667–1669, 1978.
5. Gelboin, H. V., and Ts'o, P. O. Polycyclic Hydrocarbons and Cancer. New York: Academic Press, Inc., 1978.
6. Gold, A. Carbon black adsorbates: separation and identification of a carcinogen and some oxygenated polyaromatics. Anal. Chem., *47*: 1469–1472, 1972.
7. Gold, A., Brester, J., and Eisenstadt, E. Synthesis of cyclopenta(*cd*)pyrene 3,4-epoxide, the ultimate mutagenic metabolite of the environmental carcinogen cyclopenta(*cd*)pyrene. J. Chem. Soc. (Chem. Commun.), 903–904, 1979.
8. Gold, A., Schultz, J., and Eisenstadt, E. Relative reactivities of pyrene ring positions: cyclopenta(*cd*)pyrene via an intramolecular Friedel-Crafts acylation. Tetrahedron Lett., 4491–4494, 1978.
9. Gold, A., Schultz, J., and Eisenstadt, E. Synthesis and metabolism of cyclopenta(*cd*)pyrene. *In:* P. W. Jones and P. Leber (eds.), Polynuclear Aromatic Hydrocarbons, pp. 695–704. Ann Arbor, Mich.: Ann Arbor Science Publishers, 1979.
10. Grimmer, G. Analysis of automoblile exhaust condensates. In: U. Mohr, D. Schmähl and L. Tomatis (eds.), Air Pollution and Cancer, IARC Scientific Publication No. 16, pp. 29–39. Lyon, France: International Agency for Research on Cancer, 1977.
11. Jerina, D. M., Lehr, R. E., Yagi, H., Hernandez, O., Dansette, P. M., Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., and Conney, A. F. Mutagenicity of benzo(*a*)pyrene derivatives and the description of a quantum mechanical model which predicts the ease of carbonium ion formation from diol-epoxides. *In:* F. J. DeSerres, J. R. Fouts, J. R. Bend, and R. M. Philpot (eds.), *In Vitro* Metabolic Activation in Mutagenesis Testing, pp. 159–177. Amsterdam: Elsevier, North Holland Biomedical Press, 1976.
12. Kaden, D. A., Hites, R. A., and Thilly, W. G. Mutagenicity of soot and associated polycyclic aromatic hydrocarbons to *Salmonella typhimurium*. Cancer Res., *39*: 4152–4159, 1979.
13. Neal, J., and Trieff, N. M. Isolation of an unknown carcinogenic hydrocarbon from carbon blacks. Health Lab. Sci., *9*: 32–38, 1972.
14. Nesnow, S., and Heidelberger, C. The effect of modifiers of microsomal enzymes on chemical oncogenesis in cultures of C3H mouse cells lines. Cancer Res., *36*: 1801–1808, 1976.
15. Reznikoff, C. A., Bertram, J. S., Brankow, D. W., and Heidelberger, C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res., *33*: 3239–3249, 1973.
16. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res., *33*: 3231–3238, 1973.
17. Skopek, T. R., Liber, H. L., Kaden, D. A., Hites, R. A., and Thilly, W. G. Mutation of Human Cells by Kerosene Soot. J. Natl. Cancer Inst., *63*: 309–312, 1979.
18. Snook, M. E., Severson, R. E., Arrendale, R. F., Higman, H. C., and Chortyk, O. T. The identification of high molecular weight polynuclear aromatic hydrocarbons in a biologically active fraction of cigarette smoke condensate. Beitr. Tabakforsch., *9*: 97–101, 1977.
19. Wallcave, L. Gas chromatographic analysis of polycyclic aromatic hydrocarbons in soot samples. Environ. Sci. Technol., *3*: 948, 1969.
20. Wallcave, L., Nagel, D. L., Smith, J. W., and Waniska, R. D. Two pyrene derivatives of widespread environmental distribution: cyclopenta(*cd*)pyrene and acepyrene. Environ. Sci. Technol., *9*: 143–145, 1975.
21. Wood, A. W., Levin, W., Chang, R. L., Huang, M-T., Ryan, D. E., Thomas, P. E., Lehr, R. E., Kumar, S., Koreeda, M., Akagi, H., Ittah, Y., Dansette, P., Yagi, H., Jerina, D. M., and Conney, A. H. Mutagenicity and tumor-initiating activity of cyclopenta(*cd*)pyrene and structurally related compounds. Cancer Res., *40*: 642–649, 1980.